# Muscle founder cells regulate defasciculation and targeting of motor axons in the *Drosophila* embryo

Matthias Landgraf*, Mary Baylies† and Michael Bate*

**During *Drosophila* embryogenesis, motor axons leave the central nervous system (CNS) as two separate bundles, the segmental nerve (SN) and intersegmental nerve (ISN). From these, axons separate (defasciculate) progressively in a characteristic pattern, initially as nerve branches and then as individual axons, to innervate target muscles [1,2]. This pattern of branching resembles the outgrowth and defasciculation of motor axons from the neural tube of vertebrate embryos. The factors that trigger nerve branching are unknown. In vertebrate limbs, the branched innervation may depend on mesodermal cues, in particular on the connective tissues that organise the muscle pattern [3]. In *Drosophila*, the muscle pattern is organised by specific mesodermal cells, the founder myoblasts, which initiate the development of individual muscles [4–6]. Founder myoblasts fuse with neighbouring non-founder myoblasts and entrain these to a specific muscle programme, which also determines their innervation [4,7]. In the absence of mesoderm, ISN and SN can form, but motor axons fail to defasciculate from these bundles [7]. The cue(s) for nerve branching therefore lie within the mesoderm, most likely in the muscles and/or in the precursor cells of the adult musculature [8]. Here, we show that founder myoblasts are the source of the cue(s) that are required to trigger defasciculation and targeted growth of motor axons. Moreover, we found that a single founder myoblast can trigger the defasciculation of an entire nerve branch. This suggests that the muscle field is structured into sets of muscles, each expressing a common defasciculation cue for a particular nerve branch.**

Addresses: *Department of Zoology, Downing Street, Cambridge CB2 3EJ, UK. †Molecular Biology Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue Box 310, New York, New York 10021, USA.

Correspondence: Matthias Landgraf
E-mail: ml10006@cus.cam.ac.uk

Received: **6 April 1999**
Accepted: **23 April 1999**

Published: **24 May 1999**

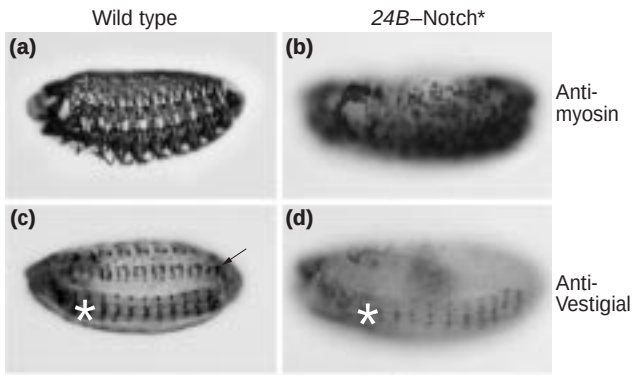
Current Biology 1999, 9:589–592
http://biomednet.com/elecref/0960982200900589

 ISSN 0960-9822

## Results and discussion

We reasoned that founder myoblasts might provide the cues for axonal defasciculation and targeted growth. To test this, we used the fact that founder-cell segregation, like the segregation of neuroblasts, depends on lateral inhibition, which requires the functions of the neurogenic genes. Loss of function of genes such as *Notch* or *Delta* leads to an overproduction of founder-like cells in the mesoderm at the expense of non-founder myoblasts [9–11]. To inhibit segregation of founder cells and thereby prevent muscle formation, we expressed activated Notch [12] throughout the mesoderm from stage 10 onwards (using the GAL4 system and *24B*GAL4 as the mesoderm-specific driver [13]). In these *24B*–Notch* embryos, the ectoderm developed relatively normally, but in the mesoderm, muscle founders failed to form (Figure 1c–d). Instead, rounded, unfused myoblasts accumulated and expressed muscle myosin but did not fuse to form muscles (Figure 1a,b). Strikingly, despite the presence of these myoblasts, the behaviour of motor axons in these embryos resembled that seen in *twist* mutant embryos, which lack mesoderm: SN and ISN grew out but axons failed to defasciculate and the nerves remained unbranched (Figure 2a,b). Because we know that myoblast fusion is

**Figure 1**




Ectopic expression of activated Notch in the mesoderm suppressed formation of founder myoblasts. Stage 16 (a,c) wild-type and (b,d) *24B*–Notch* embryos. (b) Ectopic expression of activated Notch in the mesoderm suppressed founder-myoblast segregation. Anti-myosin staining revealed that instead of syncytial muscles, seen in (a), only unfused, rounded myoblasts formed in *24B*–Notch* embryos (b). (c) Vestigial is a nuclear marker for a subset of founder cells and internal muscles (indicated by the arrow) [4,9,21]. Vestigial was also expressed in a few cells in the CNS (indicated by the asterisk). (d) In *24B*–Notch* embryos, Vestigial-positive founder cells rarely formed in the mesoderm (0.5%; n = 200), but Vestigial expression in the CNS (indicated by the asterisk) was not affected. We have obtained similar results with other founder cell markers (S59 and Even-skipped). Anterior is to the left, dorsal is uppermost.

not required for normal patterns of nerve growth [7], this experiment suggests that the founder cells are necessary for the normal defasciculation and targeted growth of the motor axons.

To show that founder myoblasts can trigger axon branching, we looked at segments of *24B*–Notch* embryos in which loss of founders was incomplete. In every case, axons defasciculated from the principal nerve trunks and innervated the remaining isolated muscles (Figure 2c,d; n = 132 muscles in 79 of 154 hemisegments). Significantly, the specificity of axon branching was maintained. As in wild-type embryos, muscles that expressed the cell adhesion molecule connectin were exclusively and reliably contacted by connectin-expressing SN axons (data not shown; n = 83 hemisegments) [14,15] and *islet-τ-myc*-expressing ISNb axons [16] exclusively contacted their target ventral muscles (n = 212 hemisegments). Moreover, in the absence of ventral target muscles, ISNb axons projected dorsally within ISN (Figure 2c–f; 98%, n = 63 hemisegments), suggesting that the default state of motor axons is growth dorsally and that ISNb axons are normally restricted to the ventral sector of the muscle field by attraction to their target ventral muscles.

Mesodermally derived precursors of the adult musculature (so-called 'persistent Twist cells' because they maintain Twist expression) have also been implicated in directing nerve branching [8] and their segregation was inhibited in *24B*–Notch* embryos. We found that nerve branching occurred in the absence of adult muscle precursors, but only in response to founder myoblasts (n = 62 in 140 hemisegments) and those adult muscle precursors that did form were rarely found at nerve branching points (Figure 3; 6%; n = 31 muscles in 140 hemisegments). We conclude that founder myoblasts not only contain the information necessary to endow each muscle with its specific characteristics, but also provide the cues that induce defasciculation and targeted growth of motor axons.

Normal defasciculation is progressive: ISNb defasciculates from ISN and individual axons defasciculate from ISNb to innervate their muscle targets [17,18]. To determine whether founder cells simply act as targets for individual axons or whether these cells can trigger the initial formation of entire nerve branches, we examined the behaviour of ISNb, using the cell adhesion molecule fasciclin III as an ISNb-specific marker [7,17]. The target muscles of ISNb, which are ventral and express Vestigial, rarely formed in our *24B*–Notch* embryos, however (0.5%, n = 200 hemisegments). We therefore studied embryos

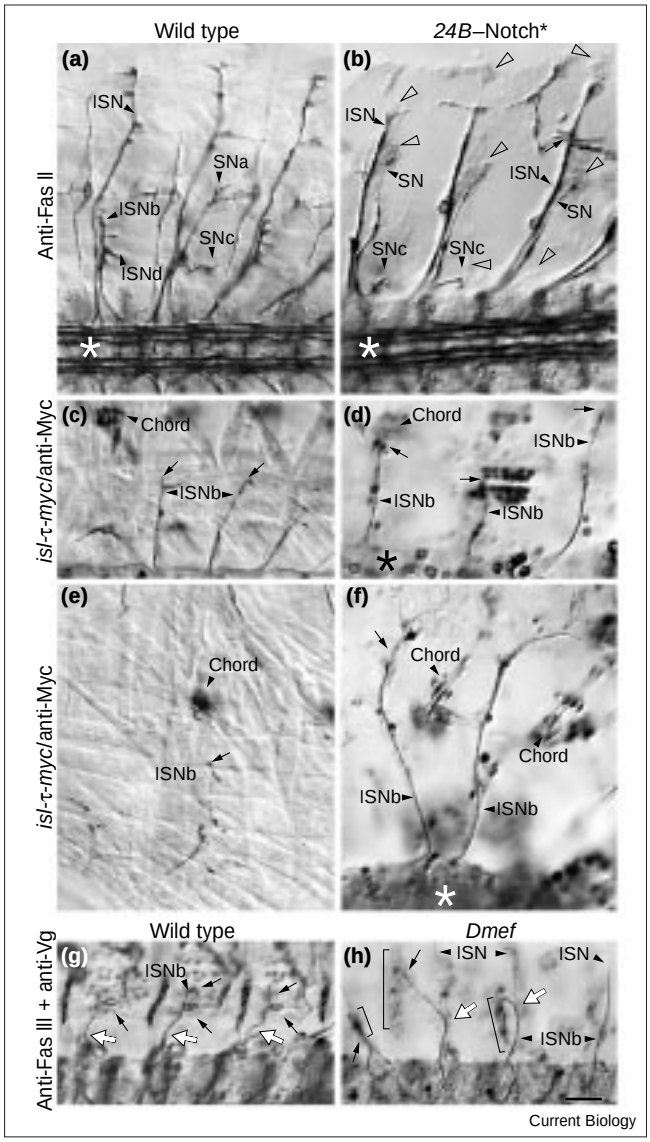
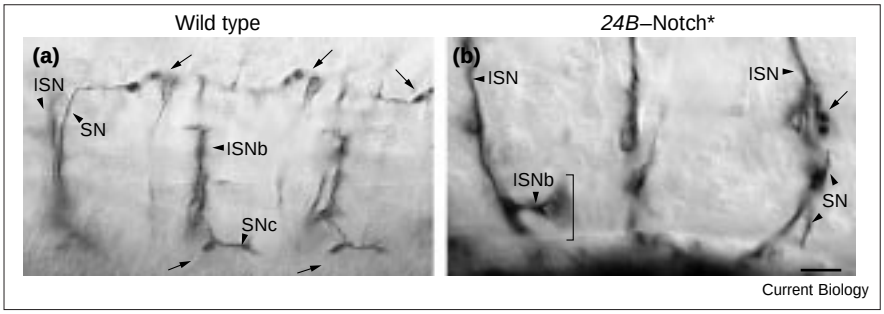



**Figure 2**

Muscle founder cells are required for peripheral nerve branching. Late stage 16 (a) wild-type and (b) *24B*–Notch* embryos stained with anti-fasciclin II (anti-Fas II) to reveal motor axons. (b) Without target muscles the main nerve trunks (ISN and SN) grow out and terminate on sensory neurons (indicated by open arrowheads), but nerve branches fail to form. In the anterior segment shown, the nerve branch SNc has formed in response to a target muscle. In the central segment shown, SNc has formed in response to ingrowing sensory axons. SNc is the only nerve branch that can do so; this was seen in 16% of segments (n = 154). (c–f) The *islet-τ-myc* (*isl-τ-myc*) reporter construct labels ISNb, the ventral branch of ISN. (d,f) Without muscles, ISNb remains fasciculated with ISN and projects past its ventral target domain by late stage 16 (d) and past the lateral chordotonal organs (Chord) into the dorsal domain by late stage 17 (f). Arrows indicate ISNb growth cones. Unexpectedly, the anti-Myc serum stains nuclei of muscles and peripheral glia in the *24B*–Notch* background. (g,h) Late stage 16 (g) wild-type and (h) *Dmef2* mutant embryos double-stained with anti-fasciclin III antibodies (anti-Fas III), which label ISNb axons, and anti-Vestigial antibodies (anti-Vg), which label the nuclei of target muscles of ISNb, indicated by brackets. Without target muscles (posterior segment), ISNb remains fasciculated with ISN. In response to even a single target founder myoblast (anterior segments), the entire ISNb defasciculates (open arrows indicate ISNb–ISN branch points). Anterior is to the left, dorsal is uppermost. The scale bar represents 15 μm. Asterisks in (a,b,d,f) indicate the CNS.

**Figure 3**

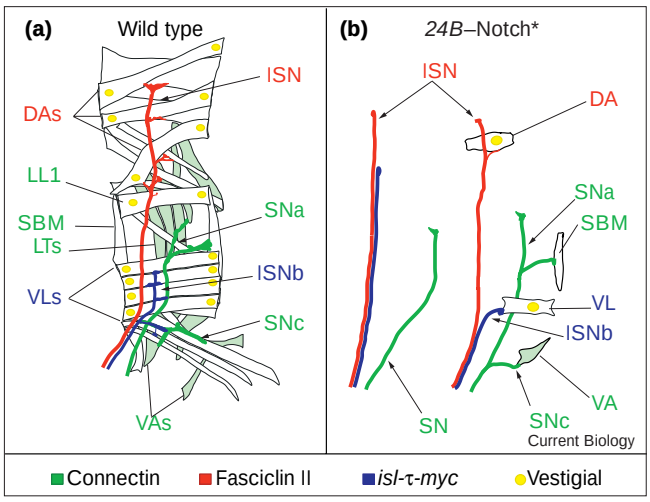



Persistent Twist cells (PTCs) are not required for nerve branching. Late stage 16 (a) wild-type and (b) *24B*–Notch* embryos double-stained with anti-fasciclin II antibodies, which label motor axons, and anti-Twist antibodies, which label nuclei. (a) PTCs (arrows) are normally associated with nerve branches. (b) Nerve branches do not form in response to PTCs (arrow in the posterior segment) and form only in response to target muscles (bracket), even in the absence of PTCs (anterior segment). Anterior is to the left, dorsal is uppermost. The scale bar represents 11 μm.

that were mutant for *Dmef2*, in which myoblast fusion fails and the pattern of ventral muscle founders is variable [19,20]: in some segments there are no founders and in others single founders or groups of founders are formed and can be identified by their expression of Vestigial [4,9,21]. In *Dmef2* mutant embryos, in the absence of ventral Vestigial-expressing founders (n = 56 of 152 hemisegments), ISNb failed to defasciculate from ISN and continued to grow dorsally, as in the *24B*–Notch* embryos. Strikingly, in these embryos, if even only a single ventral Vestigial-expressing founder cell was present (n = 85 of 152 hemisegments), ISNb defasciculated as a bundle and grew to contact the founder (Figure 2h). We conclude that muscle founders not only provide the cues for targeted growth of individual axons but also are necessary and sufficient to trigger the formation of major nerve branches. Thus, the muscle field appears to be structured into sets of muscles, each expressing a common defasciculation cue for a particular nerve branch. Within each such set, muscle targets are individually recognisable, presumably because of the expression of further cues.

In vertebrate embryos, experiments suggest that the muscle pattern is organised not by special myoblasts but by connective tissues of mesodermal origin, which are also thought to provide the necessary cues to direct the pattern of peripheral nerve branching [3,22–24]. The sequence of nerve branching also depends on reductions in the mutual adhesiveness of axons within the motor nerve (by polysialation of the neural cell adhesion molecule N-CAM) [25,26]. The way in which defasciculation is triggered, however, is not clear. In *Drosophila*, inter-axonal adhesion is mediated by a number of neural cell adhesion molecules (such as fasciclin II, fasciclin III and connectin) and is antagonised by Beaten path, a protein secreted by motor neuron growth cones and expressed at high levels in motor neurons whose axons branch from the main nerve trunks [27]. The fact that overexpression of neural cell adhesion molecules [28], like the loss of Beaten path expression [27], interferes with nerve branching, mimicking the effects of removing founder cells, indicates that the balance of adhesive forces is a decisive factor in branch formation. We found that the differential expression of *beaten path* mRNA was established before contact with target muscles and was independent of the presence of these muscles (data not shown). Mutations in *dptp69D*, *dptp99A* and *Dlar* also cause failures of nerve branching [29,30]. These genes encode receptor tyrosine phosphatases whose extracellular domains may interact with ligands provided by founder myoblasts and whose intracellular catalytic

**Figure 4**




The *Drosophila* neuromuscular system. Diagram of nerve branching and body-wall muscle innervation in (a) wild-type and (b) *24B*–Notch* embryos. Subsets of muscles and motor neurons express characteristic proteins: green, connectin; blue, *isl-τ-myc* reporter; yellow, Vestigial. All motor axons also express fasciclin II (red). Connectin-expressing muscles are innervated by connectin-positive axons. ISNb is labelled by *isl-τ-myc* and innervates Vestigial-expressing ventral longitudinal muscles. Anterior is to the left, dorsal is uppermost. Muscle nomenclature shown is as described [3]: DA, dorsal; LL, lateral longitudinal; LT, lateral transverse; SBM, segment border muscle; VA, ventral acute; VL, ventral longitudinal.

domains may regulate the growth-cone cytoskeleton in response to such interactions [31,32].

Our results suggest a model in which motor axons are sorted into distinct bundles by differential expression of *beaten path* and nerve-branch-specific homophilic cell adhesion molecules (Figure 4). Founder-myoblast-specific cues act, possibly through receptor tyrosine phosphatases, to trigger local changes in general interaxonal adhesion. These changes alter the balance of forces, allowing specific groups of axons to respond preferentially to cues in the muscle field, thereby triggering the formation of a branch.

## Materials and methods

The fly stocks that were used were Oregon-R and *Df(2R)P520/mef2*$^{22-21}$ (courtesy of H.T. Nguyen; [19]). The *isl-τ-myc* reporter construct, which labels ISNb axons [16], was kindly provided by S. Thor. We used *24B*GAL4 [13] as a mesoderm driver (at 29°C) for *24B*–Notch* (UAS*N*$^{Intra1790}$ [12]; courtesy of T. Lieber). Antibody stainings were performed as previously described [4]; the mouse anti-connectin monoclonal antibody C1.427 (kindly provided by R. White [14]) was diluted 1:10, the mouse anti-fasciclin II monoclonal antibody 1D4 and the mouse anti fasciclin III monoclonal antibody 2D5 (courtesy of C. Goodman [8,33]) were both diluted 1:5, the rabbit anti-myosin antibody (kindly provided by D. Kiehart [34]) was diluted 1:500, the mouse anti-Myc antibody (Oncogene Science) was diluted 1:200, and the rabbit anti-Vestigial antibody (kindly provided by S. Carroll [21]) was diluted 1:1000.

## Acknowledgements

We are indebted to Toby Lieber for use of the UAS*N*$^{Intra1790}$ flies prior to publication. We also thank A. Brand, S. Carroll, C. Goodman, D. Kiehart, H.T. Nguyen, J.B. Thomas, S. Thor, R. White and R.A. Schulz for the generous supply of antibodies and fly stocks. This work was funded by a studentship from the Sir Halley Stewart Trust and from the Medical Research Council to M.L. and by grants from the Wellcome Trust to M. Bate.

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